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(71) Applicants *(for all designated States except US)*: WASHINGTON UNIVERSITY [US/US]; 724 South Euclid, Box 8013, St. Louis, MO 63110 (US). THE JEWISH HOSPITAL OF ST. LOUIS [US/US]; 216 South Kingshighway, St. Louis, MO 63110 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: SCHWARTZ, Alan, L. [US/US]; 11 Southmoor Drive, Clayton, MO 63105 (US). WARSHAWSKY, Ilka [US/US]; 232 North Kingshighway Boulevard #913, St. Louis, MO 63108 (US). BROZE, George, J. [US/US]; 15 West Point Lane, Ladue, MO 63131 (US).

(74) Agents: BENNETT, Dennis, A. et al.; G.D. Searle & Co., Corporate Patent Dept., P.O. Box 5110, Chicago, IL 60680-5110 (US).

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(54) Title: METHODS AND COMPOSITIONS FOR INHIBITION OF HEPATIC CLEARANCE OF TISSUE FACTOR PATHWAY INHIBITOR

(57) Abstract

The present invention discloses methods and compositions for inhibiting the hepatic clearance of Tissue Factor Pathway Inhibitor (TFPI).

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METHODS AND COMPOSITIONS FOR INHIBITION OF HEPATIC CLEARANCE OF TISSUE FACTOR PATHWAY INHIBITOR

Field of the Invention

This invention relates to methods and compositions for inhibiting the hepatic clearance of Tissue Factor Pathway Inhibitor (TFPI).

Background of the Invention

Tissue factor (TF)[1] is a 45-kDa integral membrane glycoprotein that is an essential cofactor in initiating the extrinsic pathway of blood coagulation. In response to blood vessel injury, TF, which is produced constitutively by cells that are separated from blood by the vascular endothelium, gains access to the plasma. Plasma factor VII or VIIa binds TF and the resulting factor VIIa-TF complex activates factors X to Xa and IX to IXa. This eventually leads to the generation of thrombin and the formation of a fibrin clot. TF-induced blood coagulation is primarily regulated by tissue factor pathway inhibitor (TFPI), a 42-kDa plasma glycoprotein also referred to as lipoprotein-associated coagulation inhibitor (LACI) and extrinsic pathway inhibitor (EPI). TFPI contains an acidic amino-terminal domain followed by three tandem Kunitz-type protease inhibitory domains and a basic carboxy-terminal domain. Inhibition of TF-induced blood coagulation by TFPI involves a two step reaction leading to the formation of a quaternary factor Xa-TFPI-factor VIIa-TF complex. In the first step factor Xa binds to the second Kunitz domain of TFPI and in the second step, TFPI-factor Xa binds to the TF-factor VIIa complex through an interaction between the first Kunitz domain of TFPI and factor VIIa (reviewed in Refs. 1,2).

A wide range of plasma TFPI concentrations is found in normal individuals with a mean of ~2.5 nM (3). Greater than 90% of this TFPI is bound to lipoproteins (low density lipoprotein>high density lipoprotein>very low density lipoprotein), (4,5). Plasma TFPI levels increase several fold following the infusion of heparin (6,7). TFPI is thought to be released from the vascular endothelium where it may be bound to heparan sulfate or glycosaminoglycans.

Several animal studies have shown that recombinant TFPI is effective against TF-induced coagulopathy (8), prevents arterial thrombosis (9), and reduces mortality from bacterial septic shock (10). Pharmacokinetic studies (11) following an intravenous bolus injection of recombinant TFPI in rabbits have shown that TFPI clearance from the plasma is a biphasic process with half-lives of 2.3 min and 79 min. The primary organs involved in TFPI clearance are the liver and kidney (especially the outer cortex).

The low density lipoprotein receptor-related protein/a_2-macroglobulin receptor (LRP) and glycoprotein 330 (gp330) are two members of the low density lipoprotein receptor family involved in the endocytosis of several circulating plasma proteins. The endocytic function of LRP appears to be predominantly in the liver whereas that of gp330 is in the kidney (reviewed in Ref. 12). LRP and gp330 bind similar ligands including complexes between plasminogen activator inhibitor type 1(PAI-1) and tissue-type (t-PA) or urokinase-type (u-PA) plasminogen activators, ß-migrating very low density lipoproteins (ßVLDL) complexed with apolipoprotein (apo) E, lipoprotein lipase, and lactoferrin (13-15). In addition, LRP binds α_2-macroglobulin-protease complexes (α_2m*) (16-18) and *Pseudomonas* exotoxin A (19). A 39-kDa protein, also termed receptor-associated protein (RAP), copurifies with both LRP and gp330 (16,20). This 39-kDa protein is a potent inhibitor of all known ligand

interactions with LRP and gp330, as shown by ligand blotting experiments and by binding and uptake experiments in cultured cells (13,14,19,21-24). It has been recently reported that intravenous administration of the 39-kDa protein to rats prolonged the plasma half-life of t-PA from 1 min to ~6-9 min. It was also found that the 39-kDa protein itself was rapidly cleared from the circulation, with the liver and outer cortex of the kidney being the primary sites of clearance (25). Although the *in vivo* physiological role of the 39-kDa protein at present is not clear, it has been postulated to function as a regulator of LRP and gp330 activity.

Summary of the Invention

The present invention discloses a novel method of inhibiting the hepatic clearance of TFPI *in vivo* , preferably in humans, by the administration of the receptor-associated protein (RAP) or a fragment thereof to a patient receiving treatment with TFPI.

The present invention also provides a pharmaceutical composition which includes TFPI and RAP.

It is an object of the present invention to significantly increase the plasma half life of TFPI.

Another object of the present invention is to reduce the amount of TFPI administered to a mammal or patient in need of TFPI and still achieve the needed physiological results as would be achieved using a higher dosage of TFPI. By lowering the amount of TFPI needed a cost saving can be realized and the patient will be less likely to suffer from any possible adverse reactions to TFPI.

Brief Description of the Drawings

FIG. 1. 12.5% SDS-PAGE of recombinant TFPI and ^{125}I-TFPI. *Lane* 1. 10 µg of purified TFPI was applied to a SDS-polyacrylamide gel and stained with Coomassie brilliant blue. *Lane* 2. ~20,000 cpm of ^{125}I-TFPI was

subjected to SDS-PAGE. The gel was dried and exposed to film for 18 h. Molecular weight markers in kDa are indicated on the *left*.

FIG. 2. Inhibition of ^{125}I-TFPI degradation by the 39-kDa protein on rat hepatoma cells. A. Cells were incubated at 37°C for 4 h with 0.6 nM ^{125}I-TFPI in the absence or presence of increasing concentrations of the 39-kDa protein. Thereafter, buffers overlying the cell monolayers were subjected to trichloroacetic acid precipitation, and trichloroacetic acid-soluble radioactivity, representing degraded ligand, was determined. TFPI degraded in the absence of any 39-kDa protein was defined as 100%. Each *symbol* represents the average of duplicate determinations. B. Cells were incubated with 0.6 nM ^{125}I-TFPI at 37°C for the indicated periods of time in the absence (E) or presence (J) of 500 nM 39-kDa protein. At the indicated times, buffers overlying the cell monolayers were subjected to trichloroacetic acid precipitation. The radioactivity was normalized to femtomole equivalents of TFPI calculated from the specific activity of ^{125}I-TFPI. Each *symbol* represents the average of duplicate determinations.

FIG. 3. Binding of ^{125}I-TFPI to rat heptoma cells. A. Cells were incubated for 2 h at 4°C with increasing concentrations of ^{125}I-TFPI in the absence or presence of excess unlabelled TFPI or the 39-kDa protein. Total binding in the absence (J) or presence (E) of 500 nM 39-kDa protein was determined. Nonspecific binding (H) was determined in the presence of a >400-fold molar excess of unlabelled TFPI. Specific binding (B) was derived as the difference between total and nonspecific TFPI binding. *Symbols* represent the means of duplicate determinations. *Inset*, Scatchard plot of specific binding. B, bound ^{125}I-TFPI; B/F, bound/free ^{125}I-TFPI. B. Inhibition of

^{125}I-TFPI binding by unlabelled TFPI. Binding of ^{125}I-TFPI (0.6 nM) was performed in the absence or presence of increasing concentrations of unlabelled TFPI. Each *symbol* represents the average of duplicate determinations.

FIG. 4. Distribution of ^{125}I-TFPI during a single cycle of endocytosis in rat hepatoma cells in the absence and presence of the 39-kDa protein. Cells were incubated with 0.6 nM ^{125}I-TFPI in the absence or presence of 500 nM unlabelled TFPI or 500 nM 39-kDa protein for 2 h at 4°C. After washing to remove unbound ligand, cells were incubated at 37°C for selected intervals with 200 nM unlabelled TFPI in the absence or presence of 500 nM 39-kDa protein. The overlying media was removed and the cells were chilled on ice and rinsed prior to treatment with Pronase. A. Cell-surface ^{125}I-TFPI (Pronase-sensitive) in the absence (■) and presence (H) of the 39-kDa protein was determined. Dissociated ^{125}I-TFPI (tricholoracetic acid-precipitable) in the absence (■) and presence (B) of the 39-kDa protein was quantified. B. Degraded ^{125}I-TFPI (trichloroacetic acid-soluble) in the absence (E) and presence (J) of the 39-kDa protein is indicated. Absolute amounts of cell-surface, dissociated, and degraded ligand were normalized to femtomole equivalents of TFPI calculated from the specific activity of ^{125}I-TFPI. *Symbols* represent the specific signals (difference in the absence and presence of unlabelled ligand) and are the means of duplicate determinations.

FIG. 6. Inhibition of ^{125}I-TFPI degredation by 39-kDa protein constructs on rat hepatoma cells. Cells were incubated at 37°C for 4h with 0.6^{nM125}I-TFPI in the absence or presence of increasing concentrations of GST/1-319 (H), GST/115-319(B), 1-319(J), and as a negative control, GST (■). Thereafter, buffers overlying

the cell monolayers were subjected to trichloracetic acid precipitation, and trichloroacetic acid-soluble radioactivity, representing degraded ligand, was determined. ^{125}I-TFPI degraded in the absence of any competitor protein was defined as 100%. Each symbol represents the average of duplicate determinations.

FIG. 6. Inhibition of ^{125}I-TFPI degradation by anti-LRP IgG on human heptaoma cells. Cells were incubated with 0.6 nM ^{125}I-TFPI in the absence or presence of 500 nM unlabelled TFPI at 4°C for 2 h to allow surface binding. After washing, cells were incubated at 37°C for selected intervals with 200 nM unlabelled TFPI in the absence or presence of 5 µM anti-LRP IgG, 5 µM nonimmune IgG or 500 nM 39-kDa protein. The overlying buffer was then removed and precipitated with trichloroacetic acid. Degraded ^{125}I-TFPI in the absence (J) or presence of anti-LRP IgG (■), nonimmune IgG (▲), and the 39-kDa protein (E) was determined. *Symbols* represent the specific signals (difference in the absence and presence of unlabelled TFPI) and are means of duplicate or triplicate determinations.

Detailed Description of the Invention

The present invention is the discovery that RAP reduces the hepatic clearance of TFPI. By binding to the hepatic clearance receptor for TFPI, RAP prevents TFPI from being bound to the receptor and removed from the circulating plasma and degraded via endocytosis. This increases the plasma half-life of TFPI thereby prolonging TFPI's therapeutic effectiveness. An increase in the plasma half-life of TFPI means that a smaller amount of TFPI may be used, which reduces the risk of adverse reaction to TFPI. Since TFPI is very expensive to produce, a significant cost savings can be achieved

which, in turn, increases the availability of TFPI for clinical use.

RAP is an active, effective, competitive binding agent for the hepatic receptor for TFPI. This TFPI hepatic clearance-inhibiting protein is characterized by binding to LRP and inhibiting cellular degradation of TFPI via LRP up to 80-90%. Fragments of this 39kDa protein, particularly a 28kDa protein fragment, also inhibit TFPI cellular degradation. When RAP or a TFPI-hepatic clearance inhibiting fragment thereof is employed in the present invention, the standard dose of TFPI can be reduced.

Hepatic clearance of TFPI is inhibited *in vivo* in humans by administering a TFPI-hepatic clearance-inhibiting amount of RAP or a TFPI-hepatic clearance-inhibiting fragment thereof. The mode of administration is preferably intravenous. The preferred amount of RAP or fragment thereof administered to the human to inhibit hepatic clearance is in the range of about 60 to 6,000 mg/kg of body weight/dose. When the fragment of RAP is the 28kDa protein, the preferred amount administered to the human to inhibit hepatic clearance is in the range of about 38 to 3,800 mg/kg of body weight/dose. RAP or its fragments may be administered to the human concurrently with TFPI, but is preferably administered up to 20 minutes prior to the administration of TFPI. The reduction in hepatic clearance is measured by the increase in plasma half-life of TFPI.

It is to be understood that modified forms of RAP and fragments thereof which inhibit hepatic clearance of TFPI that are made by chemically or genetically modifying the amino acid sequence of RAP or fragments thereof are encompassed within the scope of the present invention. Such modified forms are characterized by their ability to

bind to LRP and to reduce hepatic clearance of TFPI between 20 and 100%.

RAP, the 39kDa protein of the present invention, has the following amino acid sequence:

```
1               10                  20
Y S R E K N Q P K P S P K R E S G E E F R M E K L N Q L W

30                  40                  50
E K A Q R L H L P P V R L A E L H A D L K I Q E R D E L A

   60                  70                  80
W K K L K L D G L D E D G E K E A R L I R N L N V I L A K

   90                  100                 110
Y G L D G K K D A R Q V T S N S L S G T Q E D G L D D P R

    120                 130                 140
L E K L W H K A K T S G K F S G E E L D K L W R E F L H H

        150                 160                 170
K E K V H E Y N V L L E T L S R T E E I H E N V I S P S D

          180                 190                 200
L S D I K G S V L H S R H T E L K E K L R S I N Q G L D R

            210                 220                 230
L R R V S H Q G Y S T E A E F E E P R V I D L W D L A Q S

              240                 250                 260
A N L T D K E L E A F R E E L K H F E A K I E K H N H Y Q

                270                 280
K Q L E I A H E K L R H A E S V G D G E R V S R S R E K

290                 300                 310
```

H A L L E G R T K E L G Y T V K K H L Q D L S G R I S R A R

320

H N E L [SEQ.ID.NO. 1]

Other aspects of the present invention are therapeutic compositions for treating the conditions referred to above. Such compositions comprise a therapeutically effective amount of TFPI and RAP in a mixture with a pharmaceutically acceptable carrier. This composition can be administered either parenterally, intravenously or subcutaneously. When administered, the therapeutic composition for use in this invention is preferably in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such a parenterally acceptable protein solution, having due regard to pH, isotonicity, stability and the like, is within the skill of the art.

MATERIALS AND METHODS

Materials. Iodo-Gen was purchased from Pierce Chemical Co. [^{125}I]iodide was from Amersham Corp. Pronase was obtained from Calbiochem. Normal rabbit (nonimmune) IgG was purchased from Sigma. Protein A-agarose was from Repligen. Tissue culture media and plasticware were obtained from GIBCO/BRL.

Protein Purification. Recombinant human full-length TFPI was produced and purified from *E. coli* (27). U.S. Patents to Broze 5,106,833, to Wun et. al. 4,966,852, and to Diaz-Collier 5,212,091 disclose methods and genes for making TFPI and fragments thereof and can be refered to for additional details on how to make TFPI and fragments thereof. The resultant protein was homogenous as determined by SDS-PAGE. In Fig.1 (*lane* 1) 10 µg of purified TFPI were electrophoresed on a SDS-polyacrylamide gel and stained with Coomassie brilliant blue. TFPI migrates as a single band with an apparent molecular weight of 35-kDa. TFPI (25-60 µg) was iodinated using the Iodo-Gen method (28). ^{125}I-TFPI had a specific radioactivity generally of 2 - 8 x 10^7 cpm/µg of protein. .The unincorporated ^{125}I after gel-filtration purification over a PD-10 column was < 2% of the total radioactivity. ^{125}I-TFPI retained 88% of its functional activity (e.g. stoichiometric inhibition of factor Xa). Fig.1 (*lane* 2) shows that ^{125}I-TFPI also migrates at 35-kDa. Recombinant glutathione S-transferase (GST)-39-kDa fusion protein and GST-39-kDa fusion protein constructs were prepared and purified as described in reference (25+26). The full-length GST-39-kDa fusion protein was cleaved with thrombin and the 39-kDa protein was purified by removing GST via heparin-agarose chromatography (25).

Antibodies. Polyclonal rabbit antibody was generated against purified human placental LRP described previously (22). Total IgG was purified using protein A-agarose.

Cell Culture. Rat hepatoma MH_1C_1 cells (21) and human hepatoma HepG2 cells (29) were cultured in Earle's minimum essential medium (with glutamine) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin (culture media). Cells were incubated at 37°C in humidified air containing 5% CO_2.

Binding and Degradation Assays. MH_1C_1 cells were seeded into 12-well dishes two days prior to assay. Cell monolayers were generally used at 80-90% confluence. The cells were washed twice with 4°C culture media and binding was initiated by adding 0.5 ml of culture media containing the indicated concentrations of ^{125}I-TFPI in the absence or presence of competitor protein (unlabelled TFPI or 39-kDa protein). After incubation for 2 h at 4°C, the cells were washed three times with culture media and lysed in 62.5 mM Tris-HCl, pH 6.8, containing 0.2% (wt/vol) SDS and 10% (vol/vol) glycerol. Radioactivity of cell lysates was determined in a Packard gamma counter. Degradation assays were performed by washing cell monolayers twice with room temperature culture media. 0.5 ml of culture media containing 0.6 nM ^{125}I-TFPI in the absence or presence of selected concentrations of the 39-kDa protein were then added to each well. After incubation at 37°C for the indicated time periods, the overlying media were removed and precipitated by the addition of bovine serum albumin to 5 mg/ml and tricholoracetic acid to 20%. Degradation of ligand was defined as the appearance of radioactive ligand fragments in the overlying media that were soluble in tricholoracetic acid.

Single cycle endocytosis assays. Cells were seeded into 12-well dishes. After washing the cells twice with 4°C culture media, 0.5 ml of culture media containing 0.6 nM ^{125}I-TFPI in the absence or presence of 500 nM 39-kDa protein were added to each well. Nonspecific binding was determined in the presence of 500

nM unlabelled TFPI. After binding for 2 h at 4°C, cells were washed three times with 4°C culture media to remove unbound ligand. Cells were then warmed rapidly to 37°C by adding prewarmed culture media containing 200 nM unlabelled TFPI (to prevent ^{125}I-TFPI rebinding) in the absence or presence of 500 nM 39-kDa protein. Following incubation at 37°C for selected intervals, the overlying medium was removed and precipitated with trichloroacetic acid. The cell monolayers were washed three times with 4°C phosphate-buffered saline (PBS) and incubated with PBS containing 0.25% Pronase for 30 min at 4°C. Cells were detached from the dishes by pipetting and separated from the buffer by centrifugation. Radioactivity of the supernatant fractions, defining cell surface ligand, was determined. Degradation of ligand was defined by the appearance of tricholoroacetic acid-soluble radioactivity in the overlying medium. The assay in HepG2 cells was similar to that in MH_1C_1 cells except cells were seeded into 24-well dishes. Cells were rinsed twice with 4°C culture media and incubated with 0.2 ml of culture media containing 5 nM ^{125}I-TFPI in the absence or presence of 500 nM unlabelled TFPI. After 2 h at 4°C cells were washed three times with 4°C culture media and rapidly warmed to 37°C by adding prewarmed culture media containing 200 nM unlabelled TFPI in the absence or presence of 500 nM 39-kDa protein, 5 µM anti-LRP IgG, or 5 µM nonimmune IgG. Following incubation at 37°C for selected intervals, the overlying medium was removed and subjected to tricholoroacetic acid precipitation.

Example I

Purification of RAP

The procedure for purification of the 39kDa protein from strains of *E.coli* carrying the over-expression plasmid pGEX-39kDa has been described in Herz, J., Goldstein, J. L., Strickland, D. K., Ho, Y. K. & Brown, M. S. (1991) *J. Biol. Chem.* (24) A modified version of that procedure, described below, was employed.

Cultures of *E.coli.* strain DH5α carrying the over-expression plasmid pGEX-39kDa were grown to mid-log phase in LB medium with 100 µg/ml ampicillin at 37°C. Cultures were cooled to 30°C and supplemented with 0.01% isopropylthio-β-D-galactoside to induce expression of the glutathione-S-transferase-39kDa fusion protein. Following a 4-6 hour induction at 30°C, cultures were cooled on ice and collected by centrifugation.

All of the following steps were carried out at 4°C. Cell pellets were lysed in PBSa containing 1% Triton X-100, 1mM pepstatin, 2.5 µg/ml leupeptin, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). Sonication of this lysate was performed using a Branson Model 450 Sonifier, with the resulting membranes and other cellular debris collected by centrifugation at 15,000 g for 30 minutes. The supernatant from this step was incubated overnight with agarose immobilized glutathione beads (Sigma Chemical Co.). The beads were then washed, and elution of the fusion protein was carried out by competition with 5 mM reduced glutathione in 50mm Tris, pH8 (Sigma Chemical Co.) Following dialysis, the fusion protein was cleaved by an overnight incubation with 100 ng of activated human thrombin per 50 µg of fusion protein. The glutathione-S-transferase epitope was subsequently removed by further incubation with agarose immobilized heparin beads.

* * * * *

The 28kDa protein fragment of RAP, has the following amino acid sequence:

1 10 20

P R L E K L W H K A K T S G K F S G E E L D K L W R E F L

30 40 50
H H K E K V H E Y N V L L E T L S R T E E I H E N V I S P
S

60 70 80
D L S D I K G S V L H S R H T E L K E K L R S I N Q G L D
R

90 100 110
L R R V S H Q G Y S T E A E F E E P R V I D L W D L A Q S
A

120 130 140
N L T D K E L E A F R E E L K H F E A K I E K H N H Y Q K
Q

150 160 170
L E I A H E K L R H A E S V G D G E R V S R S R E K H A L
L

180 190 200
209
E G R T K E L G Y T V K K H L Q K L S G R I S R A R H N E
L

[SEQ.ID.NO. 2]

The 28kDa protein is characterized by a molecular weight of 28,000 daltons on SDS-PAGE, stability to acid hydrolysis, solubility in 1% Triton X-100, and having approximately the same inhibitory activity (K_i) on TFPI cellular degradation via the hepatic receptor as the full-length 39kDa protein. The 28kDa protein has been cloned and purified as shown in the following example.

Example 2

Cloning of the 28kDa Protein

The 28kDa protein is produced with a bacterial expression system according to Warshawsky et al (26). The gene encoding this protein is synthesized using polymerase chain reaction (PCR) with the following primers:

Forward: 'CGCGTGGATCCCCCAGGCTGGAAAAGCTGTGG3' [SEQ.ID.NO. 3]

Reverse: ACGATGAATTCTCAGAGCTCATTGTGCCGAGC [SEQ.ID.NO. 4]. These PCR primers contain built-in restriction sites (BamH1 and EcoR1, respectively). The PCR product after restriction enzyme digestion is cloned directly to the pGEX-2T vector (Pharmacia). Other bacterial expression vectors may be used. The constructed plasmid is used to transform bacteria *E. coli* strain DH5α and this bacterial transformant bearing the recombinant plasmid is used to produce the 28kDa protein using the procedure of Example 1.

* * * * *

Using standard recombinant techniques, a chemically synthesized gene encoding the 28kDa protein may be prepared. The chemically synthesized gene comprises a chemically synthesized polynucleotide which codes on expression for the amino acid sequence of the 28kDa protein given above.

A 39kDa rat protein which binds to LRP and also inhibits the cellular degradation of TFPI via LRP. The 28kDa protein of this rat protein has the following amino acid sequence:

1 10 20
P R L E K L W H K A K T S G S V R L T S C A R V L H K E K

30 40 50
I H E Y N V L L D T L S R A E E G Y E N L L S P S D M T H
I

60 70 80
K S D T L A S K H S E L K D R L R S I N Q G L D R L R K V
S

90 100 110
H Q L R P A T E F E E P R V I D L W D L A Q S A N F T E K
E

120 130 140
L E S F R E E L K H F E A K I E K H N H Y Q K Q L E I S H
Q

150 160 170
K L K H V E S I G D P E H I S R N K E K Y V L L E E K T K
E

180 190 200
L G Y K V K K H L Q D L S S R V S R A R H N E L

[SEQ.ID.NO. 5]

Using standard recombinant techniques, a chemically synthesized gene encoding this rat protein may be prepared. The chemically synthesized gene comprises a chemically synthesized polynucleotide which codes on expression for the amino acid sequence of the rat protein given above.

Example 3

Inhibition of ^{125}I-TFPI degradation by 39kDa protein.

To investigate whether rat hepatoma MH_1C_1 cells were capable of mediating the cellular degradation of TFPI and to determine whether LRP was involved in this process, MH_1C_1 cells were incubated with 0.6 nM ^{125}I-TFPI in the absence or presence of the LRP-associated 39kDa protein for 4 h at 37°C. Thereafter buffer overlying the cell monolayers was subjected to trichloracetic acid precipitation and tricholoroacetic acid-soluble radioactivity, representing degraded ligand, was determined. Fig. 2A demonstrates that the 39-kDa protein inhibits ^{125}I-TFPI degradation in a dose-dependent manner with a K_i value[2] of ~100 nM. At the maximum dose of 39-kDa protein added, 1000 nM, 80% of total ^{125}I-TFPI degradation was inhibited. Similar results were obtained with human hepatoma HepG2 cells (data not shown). Fig. 2B demonstrates a time course for ^{125}I-TFPI degradation in the absence or presence of 500 nM 39-kDa protein. In the absence of the 39-kDa protein, degradation of ^{125}I-TFPI (initial concentration of 0.6 nM) increased linearly for at least 4 h. When the 39-kDa protein was included in the incubation, ^{125}I-TFPI degradation was inhibited by ~80%. These results strongly suggest that LRP mediates the cellular degradation of ^{125}I-TFPI.

Example 4

Binding of ^{125}I-TFPI to MH_1C_1 cells in the absence or presence of 39kDa protein.

To investigate whether TFPI bound to LRP prior to its uptake and degradation, saturation binding experiments were performed with ^{125}I-TFPI on MH_1C_1 cells in the absence or presence of the 39-kDa protein. Binding studies were performed at 4°C to avoid possible ligand uptake and degradation. As shown in Fig. 3A,

^{125}I-TFPI bound specifically to MH_1C_1 cells over the concentration range of 0.6-12 nM. Nonspecific binding, determined in the presence of a >400-fold molar excess of unlabelled TFPI, increased linearly and accounted for 20% of total ^{125}I-TFPI binding. Saturation of specific binding was not reached at a ^{125}I-TFPI concentration of 12 nM. Scatchard analysis (30) of the binding data yielded approximately 2 x 10^6 binding sites/cell with an apparent K_d of ~15 nM (*inset*, Fig. 3A). In Fig. 3B, the inhibition of ^{125}I-TFPI binding by increasing concentrations of unlabelled TFPI was examined. As seen, unlabelled TFPI competes with 0.6 nM ^{125}I-TFPI binding in a dose-dependent manner with a K_i value of 50 nM. The data in Figs. 3A and 3B yield an average K_d value of ~30 nM. Fig. 3A also shows that the presence of 500 nM 39-kDa protein has no apparent effect on ^{125}I-TFPI binding, indicating that the primary TFPI binding site on MH_1C_1 cells is not LRP.

Example 5

Single cycle endocytosis of ^{125}I-TFPI in the absence and presence of 39KDA PROTEIN.

To define the location of ^{125}I-TFPI during a single cycle of endocytosis in MH_1C_1 cells, the subsequent fate of a pre-bound cohort of TFPI molecules was examined. ^{125}I-TFPI was incubated with MH_1C_1 cells for 2 h at 4°C in the absence or presence of 39KDA PROTEIN to allow cell surface binding. After removal of unbound ligand, ligand uptake was initiated by incubating cells at 37°C for selected intervals with excess unlabelled TFPI (to prevent ^{125}I-TFPI rebinding) in the absence or presence of 39KDA PROTEIN. At selected intervals, the overlying media were removed and subjected to trichloroacetic acid precipitation. The cell monolayers were quickly cooled to stop further ligand internalization, rinsed, and treated with Pronase at 4°C to remove residual surface ligand. ^{125}I-TFPI bound to the cell surface underwent two fates: As seen in Fig. 4A, upon warming the cells to 37°C, approximately 50% of specifically bound ^{125}I-TFPI dissociated from the cell surface within 15 min and accumulated in the overlying media. 39KDA PROTEIN did not significantly affect this process. The rapid dissociation of TFPI from the cell surface is consistent with its' low affinity binding to MH_1C_1 cells (K_d ~30 nM). Fig. 4B demonstrates that a smaller fraction (approximately 10%) of cell surface bound ^{125}I-TFPI was taken into the cell and following a 15 min lag was degraded. 39KDA PROTEIN inhibited ~80% of this degradation.

Example 6

Inhibition of ^{125}I-TFPI degradation by 39kDa protein constructs on rat hepatoma cells

To define whether portions of the 39kDa protein were capable of inhibiting ^{125}I-TFPI degradation, a 28kDa fragment containing amino acid residues 115-319 of the

39kDa protein was generated as a fusion protein with GST. The resultant construct, GST/115-319, was examined for its ability to inhibit ^{RS}I-TFPI degradation, when compared to the full-length 39kDa protein (1-319), the full length 39kDa fusion protein (GST/1-319) or GST alone. As seen in Figure 5, the 28kDa construct is as effective as the full-length protein construct in inhibition of ^{125}I-TFPI cellular degradation

Example 7

Effect of anti-LRP IgG on the degradation of ^{125}I-TFPI by HepG2 cells.

The applicants have demonstrated that LRP mediates the cellular degradation of TFPI in hepatoma cells since this process is inhibited by both antibodies directed against LRP and by RAP. The data also suggest that while LRP mediates the cellular degradation of TFPI, the initial TFPI binding site on hepatocytes is not LRP. Two lines of evidence support this conclusion: First, RAP, an inhibitor of ligand interactions with LRP, does not inhibit TFPI binding to hepatoma cells at 4°C. MH_1C_1 cells appear to have about 10 times as many TFPI binding sites (2×10^6) as binding sites for the LRP-specific ligands t-PA (28,31), α_2M^* (Bu et al., unpublished observation), and the 39-kDa protein (31) ($0.1 - 0.5 \times 10^6$). It is possible that a small fraction of the TFPI binding sites are inhibited by RAP but this is below the sensitivity of the assay. If a fraction of cell surface TFPI binding was to LRP, this TFPI would be internalized and degraded when the cells were warmed to 37°C. Since ~10% of cell surface bound TFPI was degraded at 37°C in a RAP inhibitable manner (Fig. 4B), this may imply TFPI does bind to LRP on hepatoma cells. Alternatively it is possible that TFPI initially binds to another cell surface molecule and is transferred to LRP for uptake and degradation. A second line of evidence that suggests LRP is not the initial TFPI binding site is that the

average K_d value for TFPI binding to hepatoma cells is ~30 nM which is an approximately 10-fold lower affinity than has been observed for the binding of LRP-specific ligands to LRP. For example we reported that the K_d value for t-PA and RAP binding to LRP on MH_1C_1 cells was ~3-6 nM (28,31). Williams et al. (32) reported that the 39-kDa protein (recombinant RAP) bound to purified LRP with a K_d value of 4 nM. Moestrup and Gliemann demonstrated (33) that α_2M^* bound to purified LRP with two affinities (K_d values of 40 pM and 2 nM). It is interesting to note that Callander et al. (34), using human ovarian carcinoma cell lines, observed ~0.3 x 10^6 TFPI binding sites/cell with an average K_d value of 4.5 nM. The differences in the number of binding sites/cell and K_d value we observed from what Callander found may reflect differences in the TFPI used and/or differences in the cell lines.

Several ligands for LRP mediated endocytosis/degradation are initially bound to other cell surface molecules prior to their uptake and degradation by LRP. In monocytes (35), u-PA:PAI-1 complexes initially bind to the glycosyl-phosphatidyl- inositol anchored u-PA receptor since u-PA:PAI-1 complex binding is not inhibited by the 39-kDa protein but is inhibited by the amino-terminal fragment of u-PA, a 16-kDa portion of u-PA which binds to the u-PA receptor as well as u-PA itself (36). Following binding, u-PA:PAI-1 complexes are thought to be transferred from the u-PA receptor to LRP for internalization and degradation since this process is inhibited by both the 39-kDa protein and by polyclonal anti-LRP antibodies (35).

Heparan sulfate proteoglycans (HSPG) constitute a second class of cell-surface binding proteins that present ligands to LRP for uptake and degradation. Using CHO cell mutants deficient in or lacking cell surface HSPG and by pretreating HepG2 cells with heparinase, Ji et al. (37) demonstrated that HSPG serve as the initial

binding site for apoE-enriched βVLDL. Since LRP can mediate the uptake of apoE-enriched βVLDL (15), it has been proposed that HSPG bind apoE-enriched βVLDL on the cell surface and present these lipoproteins to LRP for internalization and degradation (37).

Lipoprotein lipase (LPL) is a triglyceride hydrolase that plays a key role in lipoprotein metabolism (38). LPL shares several of the same properties as TFPI: In plasma both LPL (39) and TFPI (4,5) are associated with lipoproteins. LPL and TFPI are both heparin binding proteins and after intravenous administration of heparin, plasma levels of both are increased several fold (6,7,40). LPL enhances the binding of lipoproteins to heparan sulfate both on the cell surface and in the extracellular matrix (41). LPL also enhances the binding of apoE-enriched lipoproteins to LRP on fibroblasts (42). LPL binds to purified LRP (23,43). However, in intact fibroblasts (43), degradation of LPL, but not surface binding, is blocked by antibodies directed against LRP. Taken together these results have suggested that HSPG are involved in presenting LPL to LRP for uptake and degradation. A similar model may exist for TFPI whereby HSPG or some other, as yet unidentified, cell surface molecule binds TFPI and presents TFPI to LRP for uptake and degradation.

In vivo many LRP specific ligands are rapidly cleared by the liver including LPL (44), α_2M* (45), apoE-enriched chylomicron remnants (46), t-PA (46), u-PA (48) and the 39-kDa protein (25). The 39-kDa protein is also cleared by the kidney where gp330 may mediate its' clearance (25). gp330 has also been implicated in the clearance of LPL (49) and u-PA:PAI-1 complexes (14). Since the sites of TFPI clearance are also the liver and kidney (11), it seems likely that TFPI clearance *in vivo* may be mediated by LRP and gp330. To test whether degradation of TFPI was mediated directly by LRP, the effect of antibodies directed against LRP on TFPI

degradation was examined. Human hepatoma HepG2 cells were used in this experiment since our LRP antibody was prepared against human LRP (22). ^{125}I-TFPI was incubated with HepG2 cells for 2 h at 4°C. Following removal of unbound ligand, ligand uptake was initiated by incubating the cells at 37°C in the absence or presence of competitor IgG (anti-LRP and nonimmune) or the 39-kDa protein. At selected intervals, the overlying media were removed and precipitated with trichloroacetic acid. As seen in Fig. 6, ^{125}I-TFPI degradation increased over 4 h. Anti-LRP IgG specifically inhibited this degradation by ~80% while nonimmune IgG had no effect. Fig. 6 also shows that the 39-kDa protein inhibited ~65% of ^{125}I-TFPI degradation in HepG2 cells.

Various other examples will be apparent to the person skilled in the art after reading the present disclosure without departing from the spirit and scope of the invention. It is intended that all such other examples be included within the scope of the appended claims.

TFPI is intended to be defined as not only as the full length molecule but also as fragments and/or variants thereof. In addition to full length TFPI and fragments thereof as has been disclosed above WO/91/02753 issued as EP patent 931201 discloses additional variants.

All references, patents or applications cited herein are incorporated by reference in their entirety as if written herein.

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Chem. **268**, 14176-14181.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Broze, George J., Jr.
Schwartz, Alan L.
Warshawsky, Ilka

(ii) TITLE OF INVENTION: Methods and Compositions for Inhibition of Hepatic Clearance of Tissue Factor Pathway Inhibitor

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Dennis A. Bennett, G.D. Searle & Co., Corporate Patent Dept.
(B) STREET: P. O. Box 5110
(C) CITY: Chicago
(D) STATE: Illinois
(E) COUNTRY: USA
(F) ZIP: 60680

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
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(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Bennett, Dennis A.
(B) REGISTRATION NUMBER: 34,547
(C) REFERENCE/DOCKET NUMBER: C-2795

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (708)470-6501
(B) TELEFAX: (708)470-6881

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 323 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Tyr Ser Arg Glu Lys Asn Gln Pro Lys Pro Ser Pro Lys Arg Glu Ser
1 5 10 15

Gly Glu Glu Phe Arg Met Glu Lys Leu Asn Gln Leu Trp Glu Lys Ala
20 25 30

Gln Arg Leu His Leu Pro Pro Val Arg Leu Ala Glu Leu His Ala Asp
35 40 45

Leu Lys Ile Gln Glu Arg Asp Glu Leu Ala Trp Lys Lys Leu Lys Leu
50 55 60

Asp Gly Leu Asp Glu Asp Gly Glu Lys Glu Ala Arg Leu Ile Arg Asn
65 70 75 80

Leu Asn Val Ile Leu Ala Lys Tyr Gly Leu Asp Gly Lys Lys Asp Ala
85 90 95

Arg Gln Val Thr Ser Asn Ser Leu Ser Gly Thr Gln Glu Asp Gly Leu
100 105 110

Asp Asp Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly
115 120 125

Lys Phe Ser Gly Glu Glu Leu Asp Lys Leu Trp Arg Glu Phe Leu His
130 135 140

His Lys Glu Lys Val His Glu Tyr Asn Val Leu Leu Glu Thr Leu Ser
145 150 155 160

Arg Thr Glu Glu Ile His Glu Asn Val Ile Ser Pro Ser Asp Leu Ser
165 170 175

Asp Ile Lys Gly Ser Val Leu His Ser Arg His Thr Glu Leu Lys Glu
180 185 190

Lys Leu Arg Ser Ile Asn Gln Gly Leu Asp Arg Leu Arg Arg Val Ser
195 200 205

His Gln Gly Tyr Ser Thr Glu Ala Glu Phe Glu Glu Pro Arg Val Ile
210 215 220

Asp Leu Trp Asp Leu Ala Gln Ser Ala Asn Leu Thr Asp Lys Glu Leu
225 230 235 240

Glu Ala Phe Arg Glu Glu Leu Lys His Phe Glu Ala Lys Ile Glu Lys
245 250 255

His Asn His Tyr Gln Lys Gln Leu Glu Ile Ala His Glu Lys Leu Arg
260 265 270

His Ala Glu Ser Val Gly Asp Gly Glu Arg Val Ser Arg Ser Arg Glu
275 280 285

Lys His Ala Leu Leu Glu Gly Arg Thr Lys Glu Leu Gly Tyr Thr Val
290 295 300

Lys Lys His Leu Gln Asp Leu Ser Gly Arg Ile Ser Arg Ala Arg His
305 310 315 320

Asn Glu Leu

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 209 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly Lys Phe
1 5 10 15

Ser Gly Glu Glu Leu Asp Lys Leu Trp Arg Glu Phe Leu His His Lys
20 25 30

Glu Lys Val His Glu Tyr Asn Val Leu Leu Glu Thr Leu Ser Arg Thr
35 40 45

Glu Glu Ile His Glu Asn Val Ile Ser Pro Ser Asp Leu Ser Asp Ile
50 55 60

Lys Gly Ser Val Leu His Ser Arg His Thr Glu Leu Lys Glu Lys Leu
65 70 75 80

Arg Ser Ile Asn Gln Gly Leu Asp Arg Leu Arg Arg Val Ser His Gln
85 90 95

Gly Tyr Ser Thr Glu Ala Glu Phe Glu Glu Pro Arg Val Ile Asp Leu
100 105 110

Trp Asp Leu Ala Gln Ser Ala Asn Leu Thr Asp Lys Glu Leu Glu Ala
115 120 125

Phe Arg Glu Glu Leu Lys His Phe Glu Ala Lys Ile Glu Lys His Asn
130 135 140

His Tyr Gln Lys Gln Leu Glu Ile Ala His Glu Lys Leu Arg His Ala
145 150 155 160

Glu Ser Val Gly Asp Gly Glu Arg Val Ser Arg Ser Arg Glu Lys His
165 170 175

Ala Leu Leu Glu Gly Arg Thr Lys Glu Leu Gly Tyr Thr Val Lys Lys
180 185 190

His Leu Gln Lys Leu Ser Gly Arg Ile Ser Arg Ala Arg His Asn Glu

195 200 205

Leu

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGCGTGGATC CCCCAGGCTG GAAAAGCTGT GG 32

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGAGCCGTGT TACTCGAGAC TCTTAAGTAG CA 32

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 203 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly Ser Val
1 5 10 15

Arg Leu Thr Ser Cys Ala Arg Val Leu His Lys Glu Lys Ile His Glu
20 25 30

Tyr Asn Val Leu Leu Asp Thr Leu Ser Arg Ala Glu Glu Gly Tyr Glu
35 40 45

Asn Leu Leu Ser Pro Ser Asp Met Thr His Ile Lys Ser Asp Thr Leu
50 55 60

Ala Ser Lys His Ser Glu Leu Lys Asp Arg Leu Arg Ser Ile Asn Gln
65 70 75 80

Gly Leu Asp Arg Leu Arg Lys Val Ser His Gln Leu Arg Pro Ala Thr
85 90 95

Glu Phe Glu Glu Pro Arg Val Ile Asp Leu Trp Asp Leu Ala Gln Ser
100 105 110

Ala Asn Phe Thr Glu Lys Glu Leu Glu Ser Phe Arg Glu Glu Leu Lys
115 120 125

His Phe Glu Ala Lys Ile Glu Lys His Asn His Tyr Gln Lys Gln Leu
130 135 140

Glu Ile Ser His Gln Lys Leu Lys His Val Glu Ser Ile Gly Asp Pro
145 150 155 160

Glu His Ile Ser Arg Asn Lys Glu Lys Tyr Val Leu Leu Glu Glu Lys
165 170 175

Thr Lys Glu Leu Gly Tyr Lys Val Lys Lys His Leu Gln Asp Leu Ser
180 185 190

Ser Arg Val Ser Arg Ala Arg His Asn Glu Leu
195 200

What is claimed is

1. A method of inhibiting the hepatic clearance of tissue factor pathway inhibitor in a mammual comprising administering an agent capable of binding to LRP.

2. The method as recited in claim 2 wherein said agent is selected from the group consisting of receptor-associated protein (RAP), a fragment of receptor-associated protein (RAP), Rat receptor-associated protein (39kDa protein), or an antibody against LRP.

3. The method of claim 1 wherein said agent is receptor-associated protein (RAP).

4. The method of claim 3 wherein said RAP has the following amino acid sequence

```
1               10                  20
Y S R E K N Q P K P S P K R E S G E E F R M E K L N Q L W

30                  40                  50
E K A Q R L H L P P V R L A E L H A D L K I Q E R D E L A

  60                  70                  80
W K K L K L D G L D E D G E K E A R L I R N L N V I L A K

   90                  100                 110
Y G L D G K K D A R Q V T S N S L S G T Q E D G L D D P R

     120                 130                 140
L E K L W H K A K T S G K F S G E E L D K L W R E F L H H

       150                 160                 170
K E K V H E Y N V L L E T L S R T E E I H E N V I S P S D

         180                 190                 200
L S D I K G S V L H S R H T E L K E K L R S I N Q G L D R
```

210 220 230
L R R V S H Q G Y S T E A E F E E P R V I D L W D L A Q S

240 250 260
A N L T D K E L E A F R E E L K H F E A K I E K H N H Y Q

270 280
K Q L E I A H E K L R H A E S V G D G E R V S R S R E K

290 300 310
H A L L E G R T K E L G Y T V K K H L Q D L S G R I S R A R

320
H N E L [SEQ.ID.NO. 1]

5. The method of claim 2 wherein said receptor-associated protein (RAP) fragment has an amino acid sequence of

1 10 20
P R L E K L W H K A K T S G K F S G E E L D K L W R E F L

30 40 50
H H K E K V H E Y N V L L E T L S R T E E I H E N V I S P S

60 70 80
D L S D I K G S V L H S R H T E L K E K L R S I N Q G L D R

90 100 110
L R R V S H Q G Y S T E A E F E E P R V I D L W D L A Q S A

120 130 140
N L T D K E L E A F R E E L K H F E A K I E K H N H Y Q K Q

150 160 170

L E I A H E K L R H A E S V G D G E R V S R S R E K H A L L

180 190 200 209
E G R T K E L G Y T V K K H L Q K L S G R I S R A R H N E L.

[SEQ.ID.NO. 2]

6. The method of claim 1 wherein said agent is administered 0 to 20 minutes before TFPI is adminisered.

7. The method of claim 1 wherein said agent inhibits TFPI hepatic clearance by between 20 and 100 percent.

8. A method of inhibiting the hepatic clearance of TFPI in a mammal comprising administering a TFPI hepatic clearance inhibiting amount of RAP or a fragment thereof.

9. A pharmaceutical composition, comprising:

TFPI,
An agent capable of binding to LRP; and

a pharmaceutical acceptable carrier.

10. The pharmaceutical composition of claim 9 wherein said agent is selected from the group consisting of receptor-associated protein (RAP), a fragment of receptor-associated protein (RAP), Rat receptor-associated protein and an antibody against LRP.

11. The pharmaceutical compostion as recited in claim 9 wherein said agent is receptor-associated protein (RAP) or a fragment thereof.

12. The pharmaceutical compostion as recited in claim 11 wherein RAP has the amino acid sequence of

37

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1               10                  20
Y S R E K N Q P K P S P K R E S G E E F R M E K L N Q L W

30                  40                  50
E K A Q R L H L P P V R L A E L H A D L K I Q E R D E L A

60                  70                  80
W K K L K L D G L D E D G E K E A R L I R N L N V I L A K

90                  100                 110
Y G L D G K K D A R Q V T S N S L S G T Q E D G L D D P R

120                 130                 140
L E K L W H K A K T S G K F S G E E L D K L W R E F L H H

150                 160                 170
K E K V H E Y N V L L E T L S R T E E I H E N V I S P S D

180                 190                 200
L S D I K G S V L H S R H T E L K E K L R S I N Q G L D R

210                 220                 230
L R R V S H Q G Y S T E A E F E E P R V I D L W D L A Q S

240                 250                 260
A N L T D K E L E A F R E E L K H F E A K I E K H N H Y Q
```

270 280
K Q L E I A H E K L R H A E S V G D G E R V S R S R E K

290 300 310
H A L L E G R T K E L G Y T V K K H L Q D L S G R I S R A R

320
H N E L [SEQ.ID.NO. 1]

13. The pharmaceutical compostion as recited in claim 10 wherein said RAP fragment has an amino acid sequence of

1 10 20
P R L E K L W H K A K T S G K F S G E E L D K L W R E F L

30 40 50
H H K E K V H E Y N V L L E T L S R T E E I H E N V I S P S

60 70 80
D L S D I K G S V L H S R H T E L K E K L R S I N Q G L D R

90 100 110
L R R V S H Q G Y S T E A E F E E P R V I D L W D L A Q S A

120 130 140
N L T D K E L E A F R E E L K H F E A K I E K H N H Y Q K Q

150 160 170
L E I A H E K L R H A E S V G D G E R V S R S R E K H A L L

180 190 200 209
E G R T K E L G Y T V K K H L Q K L S G R I S R A R H N E L.

[SEQ.ID.NO. 2]

FIG. 1

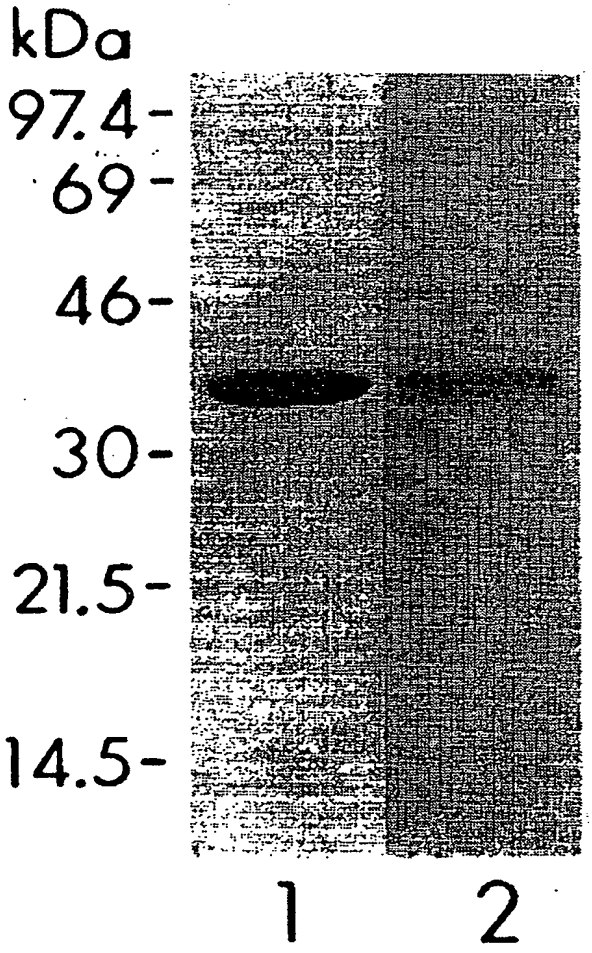


FIG. 2A

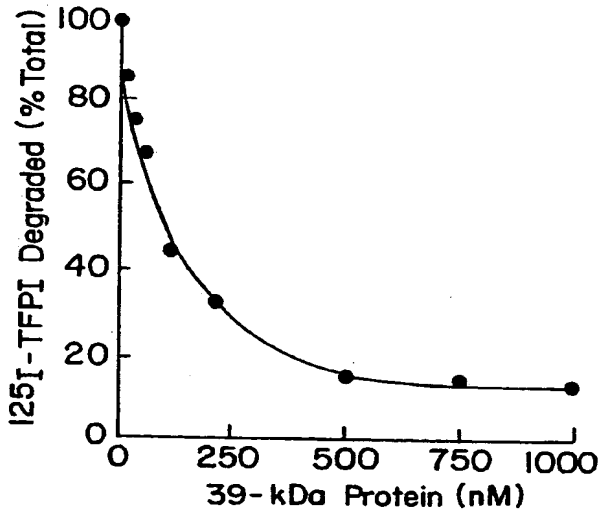


FIG. 2B

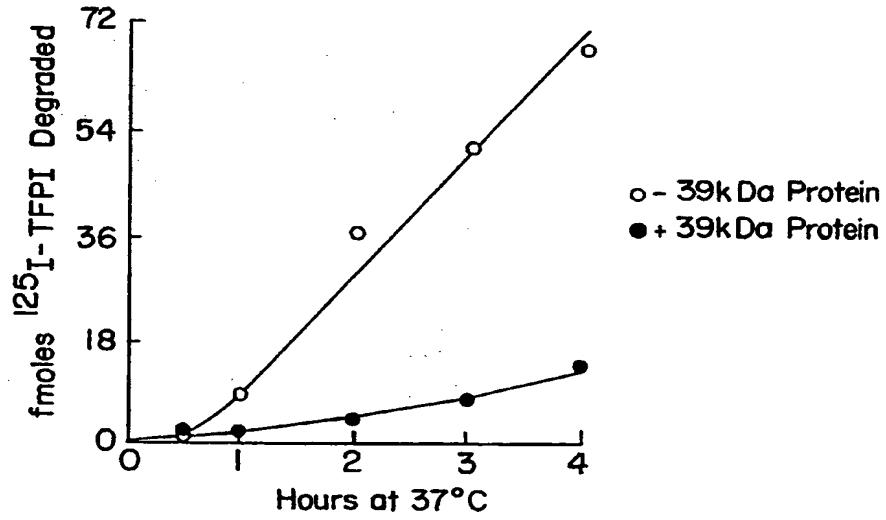


FIG. 3

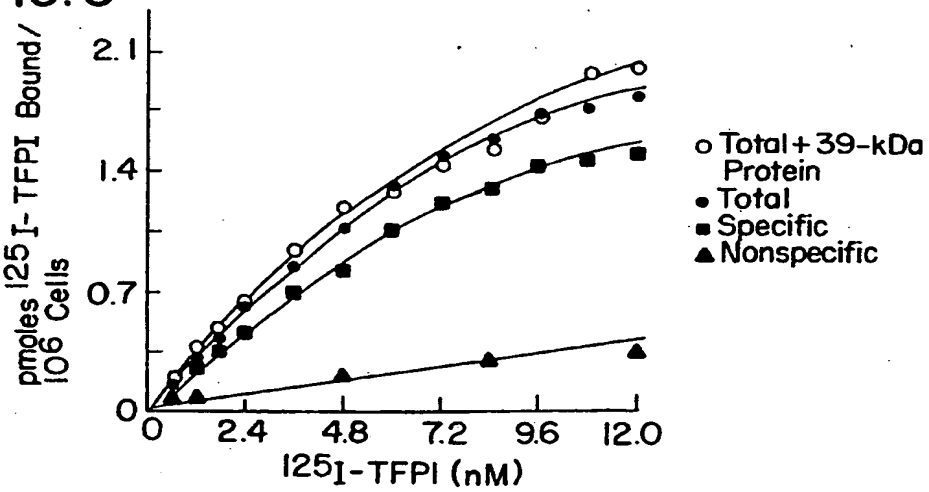


FIG. 3A

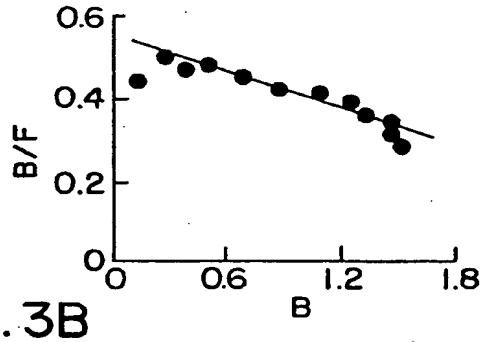


FIG. 3B

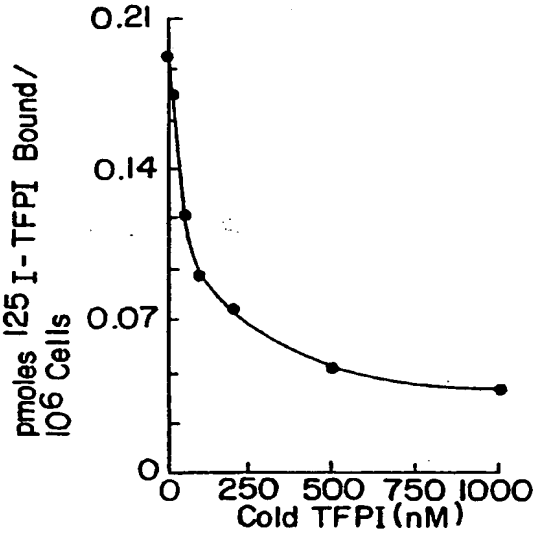


FIG. 4A

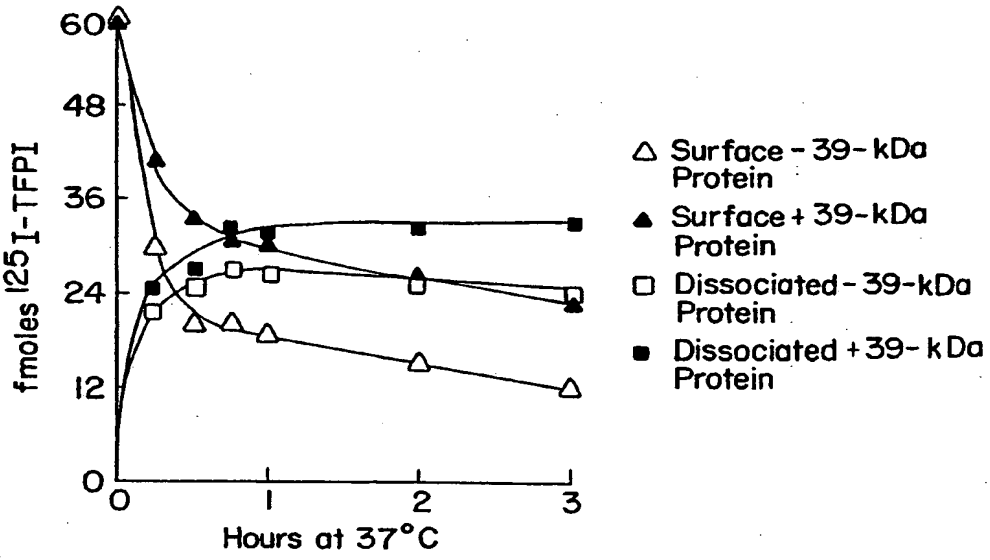


FIG. 4B

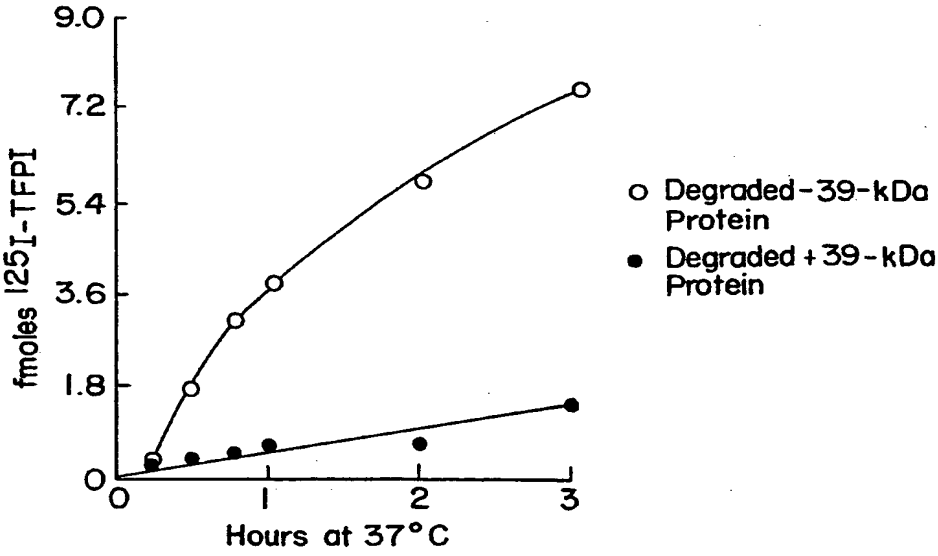


FIG. 5

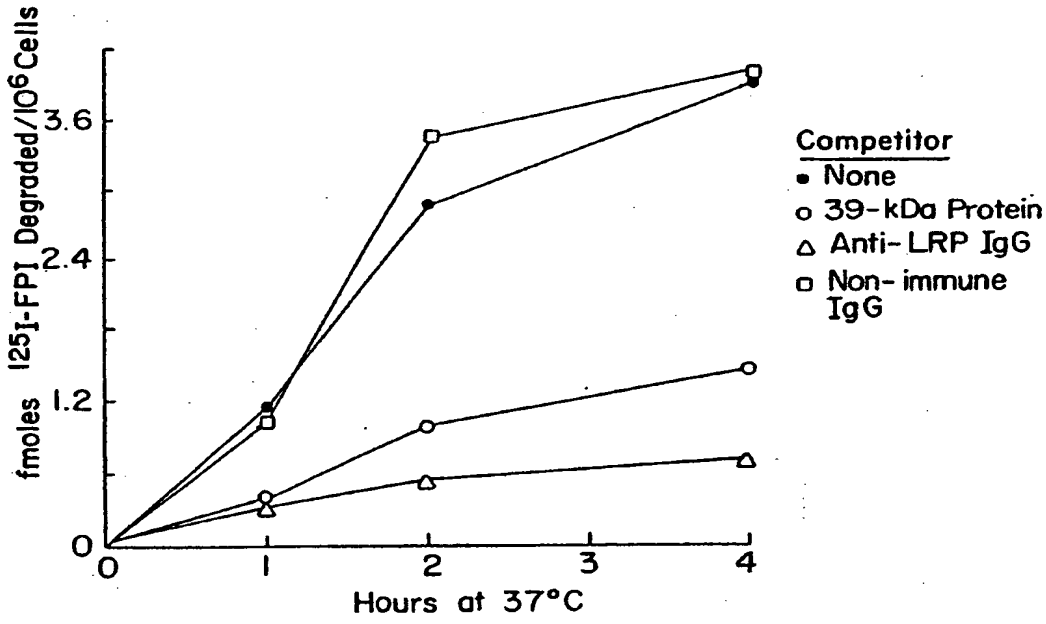
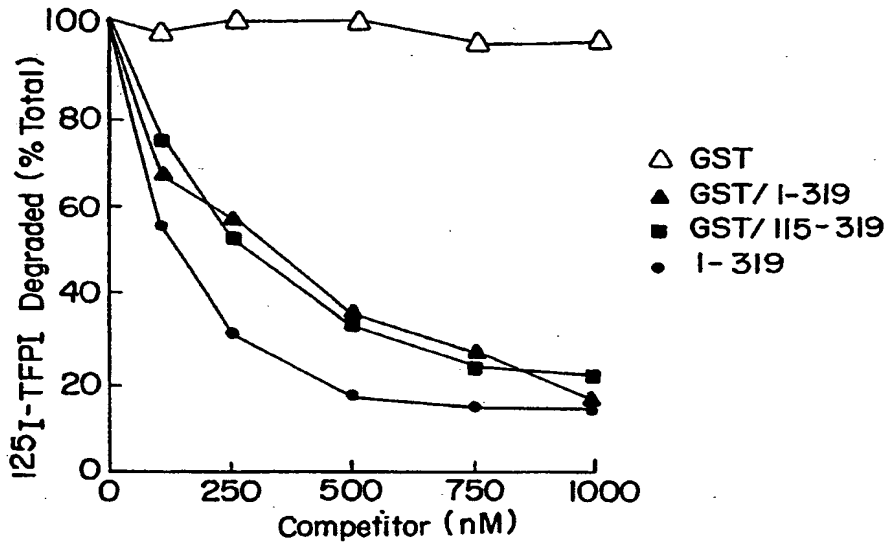

fmoles ^{125}I-FPI Degraded/10^6 Cells
3.6
2.4
1.2
0
0
1
2
3
4
Hours at 37°C
Competitor
None
39-kDa Protein
Anti-LRP IgG
Non-immune IgG

FIG.6



INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/US 95/03189

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/17 A61K38/57 //(A61K38/57,38:17)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 266, no. 20, 15 July 1991 MD US, pages 13364-13369, STRICKLAND D.K. ET AL. 'Primary Structure of alpha2-Macroglobulin Receptor-associated Protein' see the whole document ---	1-13
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 89, no. 16, 15 August 1992 WASHINGTON US, pages 7422-7426, ORTH K. ET AL. 'Complexes of tissue-type plasminogen activator and its serpin inhibitor plasminogen-activator inhibitor type 1 are ...' see the whole document ---	1-13

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

3

Date of the actual completion of the international search	Date of mailing of the international search report
22 June 1995	04 -07- 1995

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

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INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/US 95/03189

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 267, no. 29, 15 October 1992 MD US, pages 21162-21166, KOUNNAS M.Z. ET AL. 'The 39-kDa receptor-associated Protein Interacts with Two Members of the Low Density Lipoprotein Receptor Family ...' cited in the application see the whole document ---	1-13
P,X	WO,A,94 14471 (WASHINGTON UNIVERSITY) 7 July 1994 see the whole document ---	1-13
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, no. 14, 5 July 1994 WASHINGTON US, pages 6664-6668, WARSHAWSKY I. ET AL. 'The low density lipoprotein receptor related protein mediates the cellular degradation of tissue factor pathway inhibitor' see the whole document -----	1-13

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/03189

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claims 1-8 are directed to a methodof treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

INTERNATIONAL SEARCH REPORT

h. ...mation on patent family members

Internat Application No
PCT/US 95/03189

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9414471	07-07-94	AU-B- 5955494	19-07-94

Form PCT/ISA/210 (patent family annex) (July 1992)